capacitance of the supported bilayers, suggesting that it forms large pores that affect a significant fraction of the membrane surface. The increase in bilayer conductance due to MelP5 is much higher than the decrease due to either melittin or by the potent equilibrium pore-forming peptide, alamethicin. This result prompted us to develop two novel assays for macromolecule leakage from vesicles and use them to characterize MelP5, melittin and alamethicin. Under conditions where osmotic lysis does not occur, MelP5 allows the passage of macromolecules across vesicle membranes at peptide to lipid ratios as low as 1:100. Neither the melittin nor alamethicin release macromolecules significantly under these conditions. Therefore, the macromolecule-sized, equilibrium pores forms by MelP5 are unique, and MelP5 appears to belong to a novel functional class of peptide with many potential biotechnological applications.

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Peptides with the Same Composition, Hydrophobicity, and Hydrophobic Moment Bind to Phospholipid Bilayers with Different Affinities

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We investigated the dependence of membrane binding on amino acid sequence for a series of amphipathic peptides derived from δ-lysin. δ-Lysin is a 26 amino acid, N-terminally formylated, hemolytic peptide that forms an amphipathic α-helix bound at membrane-water interfaces. A shortened peptide, lysette, was derived from δ-lysin by deletion of the four N-terminal amino acid residues. Five variants of lysette were synthesized by altering the amino acid sequence such that the overall hydrophobic moment remained essentially the same for all peptides. Peptide-lipid equilibrium dissociation constants and helicities of peptides bound to zwitterionic lipid vesicles were determined by stopped-flowfluorescence and circular dichroism. We found that binding to phosphatidylcholine bilayers was a function of the helicity of the bound peptide alone and independent of the a priori hydrophobic moment or the ability to form intramolecular salt bridges. Molecular dynamics (MD) simulations on two of the peptides suggest that sequence determines the insertion depth into the bilayer. However, deeper bilayer insertion observed in the MD simulations is not synonymous with slow peptide desorption from the bilayer, as determined experimentally. We also found a systematic deviation of the experimentally determined dissociation constant and that predicted by the Wimley-White interfacial hydrophobicity scale. The reason for the discrepancy remains unresolved but appears to correlate with a predominance of isoleucine over leucine residues in the lysette family of peptides.

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Spectroscopic Investigations of Synthetic Amphiphilic Peptides in Interactions with Model Membranes

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A wide variety of organisms produce antimicrobial peptides as part of their first line of defense. These short cationic peptides are being considered as a new generation of antibiotics and represent great hopes against multiresistant bacteria which are an important clinical problem. Despite their diversity, antimicrobial peptides generally share common characteristics such as a short length of amino acids, a positive charge and an amphiphilic character. Also, it is important to note that the main target of antimicrobial peptides is the membrane(s) of pathogens. We have previously shown that a non-natural peptide composed of 14 residues (10 leucines and 4 phenylalanines modified with a crown ether) has a helical secondary structure, and is able to disrupt lipid bilayers but is not selective towards bacterial membranes. To gain specificity against negatively charged membranes, several leucines of this 14-mer have been substituted by positively charged residues (lysine, arginine, histidine). In addition, we have compared the results with those obtained with peptides substituted with negatively charged residues. Solid-state NMR experiments performed in model membranes and lipids oriented between glass plates were used to better characterize the mode of action of the charged peptides. We have also performed experiments by using the magic angle spinning technique to determine if the analogues are able to induce the segregation of anionic phospholipids. Complementary results have also been obtained by infrared and fluorescence spectroscopy. The results indicate significant differences in the membrane interactions of cationic and anionic peptides, confirming the importance of electrostatic interactions.

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Investigating the Activities of Gramicidin a in the Presence of Ionic Liquids(Ils)

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Ionic liquids(ILs) have been considered as eco-friendly solvent that can replace organic solvent due to their special properties including non-volatility, non-explosiveness, high return yield, and high manipulability. Although ILs are proven as green solvent, the micro-spills into aquatic environment are inevitable. Previous researches have already shown the toxicity of ILs to diverse organisms including bacteria and zebra fish. Our previous research has also shown that the toxicity of ILs to biological organisms may be attributed to the molecular interactions of ILs with cell membranes. However, the effects of ILs on membrane proteins have not been studied yet. The membrane proteins that play important roles in the homeostasis of biological organisms are very susceptible to the perturbation of membranes. It is important to investigate the activities of membrane proteins in the presence of ILs. In this study, we chose gramicidin A(gA) as a model membrane protein. The structure of a membrane is essential to maintain the gA dimers, thus the perturbation of membrane structure may influence the dwell time of gA dimers, resulting in the change of ion current across the membrane; gAs need to be dimerized to permeate ions through the membrane. In order to study the activities of gAs in the presence of ILs, we measured the effects of ILs on gAs using two methods: fluorescence and electrical assay. The effects of ILs increase with regard to the length of alkyl chain and the concentration of ILs. In addition, the flux of monovalent cations is hindered by the positive charge of ionic liquids. Our work suggests that ILs perturb the structure of cell membranes, giving rise to the functional changes of membrane proteins as well. We believe that our research help to design more environment friendly ILs.

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The Amantadine-Resistant S31N Mutant of Influenza a Virus M2 Protein Stably Forms a Dimer on the Living Cells

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The integral matrix protein M2 of influenza A virus has been proposed to form a pH-gated proton channel and is the target of the antiviral drug amantadine hydrochloride (Am). Am-resistant viruses (H3N2) isolated from patients during 2005−06 in the United States were screened, of which more than 90% contained the S31N mutation in the M2 protein[1]. By using fluorescence resonance energy transfer (FRET), we have demonstrated that the full-length wild-type M2 proteins (H3N2) on living cells formed a dimer at neutral pH, which was converted to a tetramer at acidic pH. In the present study, we examined the oligomeric state of the S31N mutant. The mutant exhibited a dimeric FRET signal independent of pH, whereas the S31A mutant displayed a tetrameric signal at acid pH similarly to the wild type, indicating that the S31N mutant stably formed dimers and Asn at the 31st position disturbs the arrangement of helices to inhibit tetramerization. We also found that Am did not affect both the channel activity and the oligomeric state, whereas cholesterol removal reduced the activity. These results indicate that the interaction with cholesterol allows the dimer to conduct protons and the resistance could be attributed to a change of the arrangement of helices interfering Am binding.

Reference

[1] *JAMA* (2006) **295**; 891.

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Using Spheroplasts to Study Peptide Interactions with Cell Membranes

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Cytoplasmic membranes remain intact if bacteria cells are stripped off the outer cell wall and the peptidoglycan-they are called spheroplasts or protoplasts. This allows us to study the interaction of membrane-active antibiotics directly with the cytoplasmic membranes of bacterial. For our purpose, we want giant spheroplasts in order to apply the aspiration techniques. The technique of aspiration serves two purposes: one is to apply tension to the membrane and another is to measure the membrane area changes. The measurement of the membrane area change by tension or peptide binding or any structural event is the best physical quantitative description for the state of the membrane. We believe that these

experiments will lead to discover the so-far unknown properties of cell membranes. Using established methods we grew spheroplasts which were stabilized in a STOP solution. To test the condition of the cytoplasmic membranes, we then diluted the STOP solution by adding pure water. We found that as the STOP solution was diluted, the water influx enlarged the spheroplasts. This seems to indicate that there is a membrane reservoir (perhaps, invaginations of cell membrane) in the original state of spheroplast. By the aspiration method, we measured the tension versus membrane area change of spheroplasts stabilized at 15% STOP solution. The tension vs. area change showed a slow exponential region followed by a linear region, similar to pure lipid GUVs. However the stretch expansion moduli are 5 times smaller than that of pure lipid bilayers. When maganin or melittin, were introduced into spheroplast suspension, we found that the surface area was expanded by more than 5% by peptide binding. These preliminary results indicate the feasibility of using spheroplasts as an experimental platform for studying the interaction of membrane-active peptides with cell membranes.

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High-Throughput Discovery of Peptide Antibiotics: A Delicate Balance Between Antimicrobial Potency, Solubility and Target Selectivity

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Antimicrobial peptides (AMPs) are key components of the innate immune systems of many organisms. AMPs function by permeabilizing microbial membranes, giving them an important advantage over conventional antibiotics as they may elude the selection of drug-resistance. Therefore, there has been increasing interest in engineering AMPs and improving their bioactivity over the last three decades. Yet the lack of obvious structure-function relationships or molecular design principles has obstructed the development of new AMPs. To circumvent this roadblock, we are developing a high-throughput approach to select AMPs that are optimized in all of the critical factors simultaneously. Previously in our lab, we identified a group of broad-spectrum antimicrobial peptides from a synthetic peptide library. Here, we used one of the broad-spectrum AMPs, *ARVA (RRGWALRLVLAY), to study the potency, selectivity and mechanism of action of AMPs in the presence of concentrated human erythrocytes (10⊥9 cells/ml) which mimics the in vivo milieu. *ARVA, and other AMPs, lose antimicrobial activity in concentrated erythrocytes. We developed a method to make direct measurements of peptide binding to cells which showed that loss of activity is due to weak host cell binding, coupled with the large mass excess of host cell vs. bacterial cells under physiological conditions. To identify AMPs with clinically-relevant activity, we are developing a novel, orthogonal high-throughput screen in which we select simultaneously for 1) peptide solubility; 2) lack of host cell lysis; 3) sterilization of a Gram positive microbe in the presence of concentrated erythrocytes, and 4) sterilization of a Gram negative microbe in the presence of concentrated erythrocytes. Our results show that rational library design and high-throughput screening is a promising approach to identify AMPs that have the needed balance between antimicrobial potency, solubility and target cell selectivity.

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Human Lactoferricin Derivatives as New Targeted Weapons in Cancer Therapy

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In our study derivatives of a short cationic peptide derived from the human host defense peptide Lactoferricin (LFcin) were optimized in their activity and selectivity towards cancer cells. The negatively charged membrane lipid phosphatidylserine (PS) serves as a target for these peptides since PS is specifically exposed by cancer cells (1).

Calorimetric and permeability studies showed that N-acylation and even more the repeat sequences of derivatives of hLFcin leads to strongly improved interaction with the cancer mimic PS, whereas the healthy mimic phosphatidylcholine (PC) is only slightly affected by the lipopeptide. Tryptophan fluorescence of selective peptides revealed peptide penetration only into the PS membrane interface and circular dichroism showed change of structure by increase of amount of β-sheets just in the presence of the cancer mimic. We demonstrated that hLFcin derivatives also exhibit anticancer activity *in vitro* against several cancer cell lines, correlating with selective activity against the cancer model PS. Active and selective peptides induced apoptosis only in cancer cells, whereas melanocytes and fibroblasts remained unaffected at same concentrations, yielding specificity for cancer cells higher than 100-fold for some peptides. Currently, first *in vivo* studies are under progress.

The data indicate the need of high affinity to the target PS, a minimum length and positive net charge, an adequate but moderate hydrophobicity, and capability of adoption of a defined structure exclusively in presence of the target membrane for high antitumor activity.

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(1) Riedl et al. BBA 1808: 2638-2645, 2011.

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Structure and Glycan-Binding Properties of the Vibrio Vulnificus Hemolysin B-Trefoil Lectin

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Pore forming toxins (PFTs) are secreted by bacterial pathogens as water-soluble monomers that oligomerize on the cell surface to form transmembrane channels. To increase their affinity for the membrane, many PFTs utilize proteins, carbohydrates and/or lipids as receptors. Previous studies suggest that a PFT secreted by Vibrio cholerae, V. cholerae cytolysin (VCC), recognizes cell surface glycans in its mode of action. However, while VCC contains two domains whose structures resemble known carbohydrate binding motifs, a β-trefoil and β-prism fold, only the β-prism domain actively binds carbohydrate ligands. This presents the question of why an organism would make a protein with an inactive lectin domain. Sequence analysis identified that orthologs of VCC contain either both of the lectin-like domains, or only the β-trefoil domain. Here, we utilize functional and structural approaches to investigate the carbohydrate-binding activities of a VCC ortholog, Vibrio vulnificus hemolysin (VVH), which contains only a β-trefoil lectin-like domain. Our studies identify that the VVH β-trefoil lectin domain binds a broad range of branched sugars containing multiple N-acetyllactosamine (LacNAc) repeats, with micromolar affinity. This is in contrast to the β-prism domain of VCC, which binds a narrower range of complex N-glycans with nanomolar affinity. We solved the X-ray crystal structure of the VVH β-trefoil domain identifying two interesting traits. First, the β-trefoil lectin domain alone forms a heptameric ring characteristic of many PFTs. Second, VVH contains a unique loop and coordinating residues absent in VCC. We hypothesize an evolutionary relationship between VVH and VCC such that upon gaining the more specific β-prism domain, activity of the β-trefoil domain was lost.

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Pore-Forming Peptide Toxins Obtained from Various Pseudomonas Sp. Causing Brown Blotch Disease

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Brown blotch disease is one of the most serious diseases on many cultivated mushrooms, such as *oyster mushrooms, Agaricus bisporus,* and *Flammulina velutipe*. The pathogen, identified as *Pseudomonas tolaasii,* secretes a lipodepsipeptide toxin, tolaasin. It is a pore-forming peptide toxin and causes the disease by making pores on the membranes and disrupting the cellular and fruiting body structure of mushrooms. Forty-three bacteria were isolated from the cultivated mushrooms tissues of the farms reported the outbreaks of brown blotch disease and identified by the sequence comparison of 16S rRNA genes. Five different species of *Pseudomonas*, P1-P5 subgroups, were identified. The P1 group bacteria, including 23 strains, have been identified as the main pathogen secreting tolaasin peptide. P2-P5 groups are other than *P. tolaasii* and they also cause brown blotches in pitting and cultivation tests of oyster mushroom. In the pitting test using mushroom caps, all five subtypes were able to form brown blotches. These results suggest that subgroups P2-P5 also secrete tolaasin-like peptides causing brown blotches. In order to characterize various pore-forming properties of these peptides, the effects of temperature on the toxicities of these peptide toxins were measured. The hemolytic activities of toxins from P1 to P5 subgroups were decreased by increasing temperature from 40 to 100°C. The toxicity of toxins was decreased by increasing incubation time for heat treatment. The tolaasin of *P. tolaasii*, P1 group, is an 18 amino acid-peptide, its molecular mass is 1985 Da, and it forms a left-handed α-helix. Characteristics of the peptide toxins of P2-P5 subgroups are not known. We have isolated these toxins and compared them with tolaasin by gel permeation chromatography, ion-exchange column chromatography, HPLC analysis, and mass spectroscopy.

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Cytotoxicities of Various Pore-Forming Toxins Obtained from Strains of *Pseudomonas Tolaasii*

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Pseudomonas tolaasii causes brown blotch disease on the cultivated mushrooms, such as *Agaricus bisporus* and *Pleurotus ostreatu*. It secretes a peptide